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Improved Risk Analysis by Dual Direct Detection of Total and Infectious *Cryptosporidium* Oocysts on Cell Culture in Combination with Immunofluorescence Assay▽

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The inactivation of *Cryptosporidium* oocysts is a main driver in the selection of water treatment disinfection strategies, and microbial risk analysis provides a sound basis for optimizing water treatment processes. U.S. Environmental Protection Agency method 1622/23 provides an estimate of the total oocyst count; however, it cannot be used directly for risk assessment, as it does not determine the fraction of infectious oocysts. Improved assessment of the risk for designated sources or in treated water requires evaluation of the total number of oocysts and an estimate of their infectivity. We developed a dual direct detection method using differential immunofluorescent staining that allows detection of both oocysts and cell culture infection foci for each sample. Using *Cryptosporidium parvum* oocysts, various pH levels, proteases, and gastroenteric compounds and substrates were assessed to determine their abilities to enhance the number of infection foci. The results showed that the key trigger for oocyst stimulation was acidification. Addition of a low concentration of D-glucose (50 mM) to the infection media increased rates of infectivity, while a higher dose (300 mM) was inhibitory. The total number of oocysts in each sample was determined by counting the oocysts remaining on a cell monolayer and the oocysts recovered from cell monolayer washes during processing using a simple filtration technique. With the dual direct detection on cell culture with immunofluorescence assay method, it is now possible to determine the numbers of total and infectious oocysts for a given sample in a single analysis. Direct percentages of infectivity are then calculated, which allows more accurate assessments of risk.

Over the past two decades, *Cryptosporidium* has been one of the most frequently identified etiologic agents associated with drinking water-borne illness outbreaks in the United States (8) and England and Wales (41), and it has been associated with 23.7% of protozoan outbreaks worldwide (23). In response to the emergence of this pathogen, increasingly stringent regulations regarding the removal of *Cryptosporidium* oocysts have been promulgated in the United States (44–46). The requirements for removal of *Cryptosporidium* during treatment are based on estimates of the total number of oocysts in raw water, and the average annualized incremental treatment costs associated with these requirements have been estimated to range from $92 million to $133 million (45). U.S. Environmental Protection Agency (USEPA) method 1622/23 (46) for the detection of *Cryptosporidium* in water provides a total oocyst count, but it is not ideal for characterization of risk aimed at optimizing water treatment from both the infrastructure investment and public health protection perspectives (45). Method 1622/23 does not determine whether the oocysts detected are capable of infecting humans or if they are infectious. In the risk analysis conducted by the USEPA, the proportion of the oocysts measured by the Information Collection Rule Supplement Surveys (ICRSS) that were estimated to be infectious was described by a triangular distribution (minimum, 30; maximum, 50), a method which is considered to be a source of uncertainty in evaluating risk. The USEPA estimate was based on the physical structure of the oocysts observed using method 1622 and research data from cell culture oocyst infectivity studies and represented the most likely proportion of environmental oocysts that were infectious (45). When method 1622/23 data are used, the distribution has a mode of 40%, which is supported by the infectious fraction (37%) determined by LeChevallier et al. (27) and the USEPA (45). However, the proportion of infectious environmental oocysts may vary widely from site to site. Between 4 and 20% of surface water samples tested positive for this pathogen (10, 27, 35), while approximately 40% of wastewater samples tested positive (18). In a survey of filtered, finished drinking water using a cell culture assay, 1.4% of samples tested positive for infectious *Cryptosporidium*, and for 27% (22 of 82) of the treatment plants surveyed there was at least one positive sample (1).

Cryptosporidium is an obligate parasite that travels from host to host in the form of an oocyst. Once the organism is ingested by a human via contaminated food or water or via direct contact, the body temperature and stomach acidity (pH 1.5 to 3.5) trigger oocyst excystation (24). The oocysts are then carried to the small intestine, where the chyme is alkalanized at pH 8. Bile salts secreted at this point could also promote excystation (24). Trypsin, the precursor of which is secreted by the pancreas, then enters the small intestine. Trypsin in an

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acidified buffer is considered to be a suitable *in vitro* excystation treatment (20) and a stimulant of sporozoite motility (42). In the small intestine, excysted sporozoites colonize enterocytes, forming parasitophorous vacuoles. Bile salts, especially sodium taurocholate, appear to enhance invasion of sporozoites by boosting their proteinic secretions and their gliding motility (14).

The methods available to estimate oocyst infectivity vary considerably in their oocyst stimulation and infection conditions, as well as in their application (e.g., water, wastewater, method development, biological study, disinfection assay, drug assay, etc.) (Table 1). Ice-cold bleach and acidified Hanks' balanced salts buffer (AHBSS) supplemented with trypsin at body temperature are used as stimulation media. Bile or its salts have also been used as the stimulating agent in one study, and the results compared positively to the results obtained with bleach treatment (19). Cell culture medium supplemented with 10% fetal bovine serum (FBS) is widely used. Other supplements (vitamins, bile salts, sugars, and antibiotics) can also be added. Another important variation in the methods is the infection criteria, which are dependent on the objective of the method. For example, if the objective is to quantify the impact of stimulation and the infection medium conditions on the motility or pattern of observable infection under simulated conditions, then a method analyzing life stages or focus density is used. In contrast, if the goal is to estimate the number of infectious foci, then direct focus counting or the most-probable-number (MPN) method is more suitable. However, the use of MPN with a low concentration of organisms, which is often the case with *Cryptosporidium* oocysts in environmental samples, is limited (5).

Regulatory occurrence data sets collected using method 1622/23 for drinking water supplies report total oocyst counts (43, 45, 46), but these total counts do not provide a basis on which to estimate the proportion of infectious oocysts found in the environment (10). Quantification of this fraction is needed to reduce uncertainties and improve risk analysis (45). Current analytical approaches have a substantial shortcoming as they require a given sample to be divided for multiple analysis. For example, a fraction of the sample is analyzed by an immunofluorescence detection assay to determine the total number of oocysts (e.g., method 1622/23), and a distinct fraction is analyzed by cell culture to determine infectivity (e.g., cell culture-immunofluorescence assay [CC-IFA]). Consequently, the physical division of samples results at best in indirect estimation of the proportion of infectious oocysts. For example, in one study examining surface water that used the split sample approach, 593 samples were analyzed using method 1622/23 and 560 samples were analyzed using a cell culture infectivity assay (some split samples were lost, resulting in the different numbers of samples assayed) (27). The authors reported that 60 of the method 1622/23 samples and 22 of the cell culture infectivity assay samples tested positive, resulting in an estimate that 37% of the oocysts detected were infectious. However, a Monte Carlo simulation of oocyst distribution and positive result probabilities was needed to support this estimate, since only one set of split samples tested positive by both assays.

The general objective of this study was to develop and optimize a method for combined detection of total and infectious oocysts that could be used to improve assessment of *Cryptosporidium* risk. Several different oocyst stimulation treatments and cell culture medium supplements were evaluated to optimize the *Cryptosporidium* cell culture infectivity assay. Several different commercial antibodies with different fluorochromes were evaluated individually and in combination for immunofluorescent detection of oocysts and cell culture infection foci.

MATERIALS AND METHODS

Source of *Cryptosporidium* oocysts. Viable *Cryptosporidium parvum* isolate Iowa (Harley Moon) oocysts passaged through mice were purchased from Waterborne Inc. (New Orleans, LA). All lots were used within 2 months postshedding and were stored at 4°C.

Cell culture. Human ileocecal adenocarcinoma HCT-8 cells (ATCC CCL-244) were cultivated in RPMI 1640 medium containing GlutaMAX and 25 mM HEPES buffer (Invitrogen Canada Inc., Burlington, Ontario, Canada) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Invitrogen Canada Inc., Burlington, Ontario, Canada), 0.1 g kanamycin liter^{-1}, 100,000 U penicillin G liter^{-1}, 100 mg streptomycin liter^{-1}, and 250 μg amphotericin B liter^{-1} (Calbiochem, San Diego, CA). Cells were grown in 75-cm^2 culture flasks (BD Falcon, VWR Canada) in a 5% CO_2 atmosphere at 37°C. The cells were passaged every 3 to 4 days. For infectivity studies, cells were seeded on Lab Tek II multichamber slides (catalog no. 154534; Nalge Nunc, Naperville, IL) at a target density of 2.2×10^4 cells per chamber in 500 μl cell culture medium and incubated in a 5% CO_2 atmosphere at 37°C. Within 48 h, monolayers were confluent. No cells that were passaged more than 25 times were used.

Oocyst stimulation pretreatment. Oocysts were pretreated by incubation in acidified Hanks' balanced salt solution (catalog no. H9269; Sigma-Aldrich, St. Louis, MO) at pH 2 for 1 h at 37°C with vigorous vortex mixing for 10 s every 15 min (11). In the optimization experiments, various supplements were freshly added, as follows: to the AHBSS, trypsin type II-S from porcine pancreas (catalog no. T7409; Sigma-Aldrich, St. Louis, MO) and pepsin from porcine stomach mucosa (catalog no. P7012; Sigma-Aldrich, St. Louis, MO); and to nonacidified Hanks' balanced salt solution (HBSS), bovine bile (catalog no. B3883; Sigma-Aldrich, St. Louis, MO). These supplements were selected based on the results of the studies shown in Table 1.

Infection of cell monolayers. Stimulated oocysts were washed twice using prewarmed infection medium and microcentrifugation (17,000 × *g* at room temperature for 2 min without braking). The final volumes of inocula were adjusted to 500 μl. Inoculated cell monolayers were incubated for 72 h in a 5% CO_2 atmosphere at 37°C. For the control infection, cell culture infection medium containing 10% FBS was used. To evaluate the effects on infectivity, the following supplements were added to the infection medium: D-(+)-glucose (catalog no. G8270; Sigma-Aldrich) and bovine bile (catalog no. B3883; Sigma-Aldrich). These additions were selected based on the results of the studies shown in Table 1.

Detection of infection. Infection medium was removed from each slide chamber, and monolayers were fixed with 500 μl absolute methanol (catalog no. 65542; Sigma-Aldrich, Germany) for 10 min. The methanol was removed, and the monolayers were air dried. Monolayers were then blocked for a minimum of 30 min on a rocking platform at room temperature using 500 μl phosphate-buffered saline (PBS) containing 0.002% Tween 20 (catalog no. BP337-100; Fisher Scientific, Fair Lawn, NJ) and 2% goat serum (catalog no. G9023; Sigma-Aldrich). The slides were subsequently washed with PBS twice and stained using various antibodies. The antibodies tested were Cy3-labeled Crypt-a-Glo (catalog no. A400CY3R-1X; Waterborne Inc., New Orleans, LA), Cy3-labeled Sporo-Glo (catalog no. A600CY3R-1X; Waterborne Inc., New Orleans, LA), and fluorescein isothiocyanate (FITC)-labeled EasyStain GC Combo (catalog no. EST CG80; BTF, Sydney, Australia) in 150 μl. The antibody mixture dilutions (1/10, 1/50, 1/100) and wash buffers evaluated included PBS and BTF fixing buffer from a BTF EasyStain kit. Slide chambers were removed, 2 drops of CitiFluor glycerol-PBS AF1 (catalog no. CM 512-1; Canemca Marivac, Quebec, Canada) mounting medium was added, coverslips were added, and the slides were sealed with nail polish. Monolayers were observed to determine the presence of infection foci and oocysts at a magnification of ×200 (×600 for environmental samples) using an epifluorescence microscope (BX51; Olympus, Tokyo, Japan) with FITC (U-N51006; Olympus, Tokyo, Japan) and Cy3 (U-N4107A; Olympus, Tokyo, Japan) filters.

Filtration counting of oocysts. To count oocysts in solutions or to estimate oocyst losses at various steps in the protocol, spent wash solutions were processed using the following protocol. First, spent wash solutions were microcen-

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TABLE 1. Stimulation conditions, infection media, and infection criteria used in various *Cryptosporidium* viability and infectivity studies

Reference[c]	Objective	Simulated media[a]		Infection criteria	Comments
		Stimulation	Infection		
4	Method development	AHBSS (pH 2.75), 1 h at 37°C	4 h at 37°C in either Hanks' MEM with 0.57% bovine bile and 0.06% $NaHCO_3$ or HBSS with 0.08% sodium deoxycholate and 0.18% $NaHCO_3$	Excystation, DAPI positive	Diluted acid mimics *in vivo* more, with no significant oocyst death; 0.1 M NaOH-0.1 M HCl increased oocyst death; at temperatures above 37°C, sporozoites do not survive; at temperatures below 37°C, little effect on excystation
47	Method development	10% bleach, 10 min at 4°C	RPMI 1640 with 10% FBS, 15 mM HEPES, 50 mM glucose, 3.5% ascorbic acid, 0.1% folic acid, 0.4% 4-aminobenzoic acid, 0.2% calcium pantothenate, 0.1 U/ml insulin, 100 U/ml penicillin G, 10% streptomycin, and 0.025% amphotericin B (pH 7.4)	Life stage count	Twenty-five medium supplements examined; infectivity enhancers included 10% FBS, sugars (glucose, galactose, maltose, mannose), insulin, and vitamins (ascorbic acid, calcium pantothenate, folic acid, *para*-aminobenzoic acid)
38	Method development	10.5% bleach, 10 min at 4°C	RPMI 1640 with 10% FBS, 2 mM L-glutamine, and 20 mM HEPES	Focus detection method	Detects more foci than seeded oocysts
9	Occurrence in natural water and filter backwash	AHBSS-1% trypsin, 1 h at 37°C	RPMI 1640 with L-glutamine, 10% FBS, 15 mM HEPES, 50 mM glucose, 35 mg/liter ascorbic acid, 1.0 mg/liter folic acid, 4.0 mg/liter 4-aminobenzoic acid, 2.0 mg/liter calcium pantothenate, 100,000 U/liter penicillin G, 100 mg/liter streptomycin, 700 mg/liter amphotericin B, and 12.5 mg/liter tetracycline	PCR positive	Not quantitative
32	IMS comparison	HBSS with 0.7% bile and 5 mM $NaHCO_3$, 3 h at 37°C	Eagle MEM or RPMI 1640 with 0.1% bovine serum albumin	Sporozoite infection determined by PCR detection	Oocysts excysted while in complex; sporozoite purification prior to infection
40	Method development, disinfection study	10.5% bleach, 5 to 8 min at 4°C	RPMI 1640 with 10% FBS, 2 mM L-glutamine, and 20 mM HEPES	MPN	Comparable with animal infectivity
17	Survival assay	0.5% pepsin-0.7% HCl-0.9% NaCl for 30 min at 37°C; 2.2% $NaHCO_3$-0.22% sodium taurocholate-0.04% bovine trypsin for 120 min at 37°C	Not applicable	Empty oocysts	Excystation protocol; 95% excystation obtained
28	Drug assay	Bleach	Not described	Fluorescence intensity read by enzyme-linked immunosorbent assay plate counter	Results comparable to animal assay results
19	Method development	None	RPMI 1640 with 10% FBS, 15 mM HEPES, 50 mM glucose, 3.5% ascorbic acid, 0.1% folic acid, 0.4% 4-aminobenzoic acid, 0.2% calcium pantothenate, 0.1 U/ml insulin, 100 U/ml penicillin G, 10% streptomycin, 0.025% amphotericin B (pH 7.4), and 0.375% sodium taurocholate	Parasite count at ×1,000 magnification	Sodium taurocholate better than bleach; no stimulation required with sodium taurocholate
48	Method development	10% bleach, 8 min at 4°C	RPMI 1640 with 10% FBS, Opti-MEM, and Pen-Stept-Amph	MPN and focus count	Centrifuge-stimulated oocysts on cell monolayers shorten infection time
33	Disinfection assay, comparative study	RPMI with 0.75% sodium taurocholate, 10 min at 37°C	RPMI 1640 with 4 mM L-glutamine, 2% FBS, 30 mM HEPES, 100 U/ml penicillin, 100 g/ml streptomycin, 100 g/ml kanamycin, and 0.25 g/ml amphotericin B	MPN and focus count	Incubation with sodium taurocholate increases oocyst infectivity; infectivity comparable to animal infectivity

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18	Occurrence in a wastewater treatment plant	10% bleach, 8 min at room temperature	Growth medium	MPN	
27	Occurrence in river water	AHBSS-1% trypsin, 1 h at 37°C	RPMI 1640 with L-glutamine, 10% FBS, 15 mM HEPES, 50 mM glucose, 35 mg/liter ascorbic acid, 1.0 mg/liter folic acid, 4.0 mg/liter 4-aminobenzoic acid, 2.0 mg/liter calcium pantothenate, 100,000 U/liter penicillin G, 100 mg/liter streptomycin, 700 mg/liter amphotericin B, and 12.5 mg/liter tetracycline	PCR positive	Reported CC-PCR infectivity-positive samples compared to paired samples and method 1623 positive with paired samples
30	Method development	2 mM sodium taurocholate, 10 min at 15°C, 5 to 8 min at 37°C	Not specified	Detection of infected cells by flow cytometry	Correlated with IFA visual count; less operator bias; faster
31	Occurrence in reclaimed water, comparison of method	10% bleach, 8 min at room temperature	RPMI 1640 with 10% FBS, 20 mM HEPES, 2 mM L-glutamine, and 10% Opti-MEM	FDM-MPN	Reproducible and sensitive
1	Occurrence in filtered drinking water	AHBSS-1% trypsin, 1 h at 37°C	RPMI 1640 with L-glutamine, 10% FBS, 15 mM HEPES, 50 mM glucose, 35 mg/liter ascorbic acid, 1.0 mg/liter folic acid, 4.0 mg/liter 4-aminobenzoic acid, 2.0 mg/liter calcium pantothenate, 100,000 U/liter penicillin G, 100 mg/liter streptomycin, 700 mg/liter amphotericin B, and 12.5 mg/liter tetracycline	PCR positive	Used gp60 region to differentiate environmental isolates from lab controls
20	Biological study	0.5% trypsin at pH 2.5 to 3 for 20 min at 37°C; RPMI 1640 with 0.03% L-glutamine, 0.3% Na_2CO_3, 0.02% bovine bile, 0.1% glucose, 0.025% folic acid, 0.1% 4-aminobenzoic acid, 0.050% calcium pantothenate, 0.875% ascorbic acid, 1% fetal calf serum, 0.36% HEPES buffer, 1,000 U/liter penicillin G, and 0.01% streptomycin at pH 7.4	Not applicable	Complete life cycle	Cell-free culture
10	Method development	AHBSS-1% trypsin, 1 h at 37°C	RPMI 1640 with L-glutamine, 10% FBS, 15 mM HEPES, 50 mM glucose, 35 mg/liter ascorbic acid, 1.0 mg/liter folic acid, 4.0 mg/liter 4-aminobenzoic acid, 2.0 mg/liter calcium pantothenate, 100,000 U/liter penicillin G, 100 mg/liter streptomycin, 700 mg/liter amphotericin B, and 12.5 mg/liter tetracycline	PCR positive	Faster detection; quantitative PCR detection; not for disinfection assay (background signal at high levels of oocyst inocula); standard curve not universal
35	Occurrence in surface water	AHBSS (pH 2.7) for 1 h at 37°C; 1% bovine bile-0.44% $NaHCO_3$ for 30 min at 37°C	Dulbecco modified Eagle medium with 25 mM HEPES, 45% glucose, 10% FBS, 0.1% MEM-NEAA, 0.1% L-glutamine, and 0.005% gentamicin	Foci and 50% infectious dose	Assay not sensitive enough for low environmental counts
3	Method development	AHBSS	RPMI with 0.05% bile	Focus count	Blind trial demonstrates that CC-IFA is a good surrogate for animal infectivity
21[b]	Method development	AHBSS (pH 2)-1% trypsin, 1 h at 37°C	RPMI 1640 with L-glutamine, 10% FBS, 100,000 U/liter penicillin G, 100 mg/liter streptomycin, and 700 mg/liter amphotericin B	Focus count	Reference CC-IFA protocol

[a] Abbreviations: MEM, minimal essential medium; DAPI, 4′,6′-diamidino-2-phenylindole; Pen-Stept-Amph, penicillin-streptomycin-amphotericin B; FDM, focus detection method; NEAA, nonessential amino acid.
[b] Reference method.
[c] References are listed from the oldest to the most recent studies.

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trifuged at 17,000 × *g* for 2 min. The pellets were suspended in 100 μl of a 1/50 dilution of BTF GC Combo antibodies in BTF fixing buffer for 2 h at room temperature on a rocking platform at low speed. Stained oocysts were washed once with 500 μl BTF fixing buffer and centrifuged again. The pellets were suspended in 500 μl BTF fixing buffer and vacuum filtered through a 0.45-μm polycarbonate filter (catalog no. HAWGO4756; Millipore, Billerica, MA). The tip and tube used were rinsed with 200 μl of BTF fixing buffer, and the rinse solution was passed through the filter to maximize oocyst recovery. The unused edges of the filter were removed using flame-sterilized scissors, and the filter was placed on a microscopic slide. CitiFluor glycerol-PBS AF1 (catalog no. CM 512-1; Canemca Marivac, Quebec, Canada) was used as a mounting medium. The reproducibility of staining characteristics for a 1/10 dilution of Crypt-a-Glo in PBS and a 1/50 dilution of GC Combo in BTF fixing buffer was evaluated using three replicates of the same oocyst stock suspension.

Rate of excystation of stimulated oocysts. Oocyst suspensions were subjected to a stimulation pretreatment and then were examined to determine rates of excystation. Oocysts were observed by differential interference contrast microscopy at a magnification of ×1,000. The first 75 randomly encountered oocysts were determined to be intact or excysted.

Effect of the proportion of UV-killed oocysts on cell culture infectivity and oocyst retention on monolayers. Untreated and UV-inactivated (100 mJ/cm^2; 253.7 nm; collimated beam) oocyst suspensions containing approximately 500 oocysts were mixed using different proportions (0:1, 1:4, 1:2, 3:4, 1:0). Foci and oocysts on the cell monolayers were enumerated.

Recovery with the dual direct detection (3D)-CC-IFA method. For method recovery trials and seeded environmental matrices, oocyst inocula (500 oocysts) were prepared using flow cytometry by the Wisconsin State Laboratory of Hygiene. Oocysts were added to the optimized pretreatment stimulation medium (AHBSS without trypsin). Once stimulated, the oocysts were infected in the optimized infection medium (with 50 mM glucose). Detection was performed using the Sporo-Glo stain for infection foci and BTF GC Combo for oocyst detection on cell monolayers and in spent monolayer wash solutions.

Spiked environmental assay for study of recovery from IMS purification. Grab samples of water from the Yamaska River and Mille-Iles River (Quebec, Canada) were repeatedly centrifuged at 1,500 × *g* for 15 min until one 0.5-ml pellet per immunomagnetic separation (IMS) reaction was formed. Five milliliters of supernatant was kept above the 0.5-ml pellet, and vigorous vortexing was used to homogenize the pellet. This concentrate and oocysts were added to the IMS protocol Leighton tube, and the preparation was vortex mixed vigorously. IMS capture was performed twice according to the Dynabeads anti-*Cryptosporidium* kit (catalog no. 730-01; Invitrogen Canada, Burlington, Ontario, Canada) and USEPA method 1622 protocol instructions (46). The complexes were then dissociated during the pretreatment stimulation step of the 3D-CC-IFA method, and beads were removed after 1 h of incubation at 37°C. To avoid fungal contamination, 240,000 U nystatin liter^{-1} (catalog no. N1638; Sigma-Aldrich, St. Louis, MO) was added to the infection medium.

Direct and indirect calculations of the percentage of infectious oocysts from the CC-IFA data. Data for infectious foci from either environmental samples or spiked oocysts are reported based on the corresponding total oocyst counts. The ratios could be obtained either indirectly or directly. The indirect enumeration method required a split sample (e.g., number of IMS-purified oocysts divided into the total number and the number determined by the infectious methods), and each subsample provided either a count of infectious oocysts (i.e., infectious foci) in a cell culture assay or total oocyst counts determined by USEPA method 1623. In the direct (3D-CC-IFA) enumeration method, the numbers of total and infectious oocysts were determined for a single sample in a single analysis.

RESULTS

Reference counts. Results obtained with various medium formulations were compared to results obtained with the reference method described by Johnson et al. (21) (Table 1). The reference method uses AHBSS-1% trypsin as the stimulation pretreatment and RPMI medium with 10% FBS plus antibiotics as the infection medium. When all results were compared to the results obtained with the reference method, the mean percentages of oocyst infectivity (number of foci reported for the number of oocysts inoculated) were 7.4% ± 4.0% ($n = 25$). All results were then expressed as a ratio of the number of foci to the number of foci obtained with the AHBSS-1% trypsin reference method. Also, Fig. 1 shows an overview of the whole 3D-CC-IFA method.

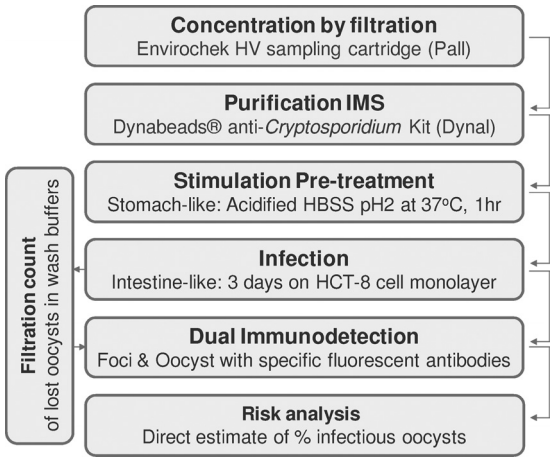

FIG. 1. Summary of the steps of the final 3D-CC-IFA method.

Stimulation pretreatment. Figure 2 shows that treatment of oocysts with trypsin at concentrations equal to or less than 1% can slightly increase the number of cell culture infection foci. At higher concentrations, a significant decrease (for 0.25 to 1% trypsin compared with 2% trypsin, $P < 0.05$) in the number of infection foci was observed. Addition of pepsin did not increase the level of infection (for 1% pepsin compared with 0.25% pepsin, $P > 0.1$), and at higher concentrations (>0.5%) the number of foci detected was significantly decreased (for 0.06 to 0.5% pepsin compared with 1 to 2% pepsin, $P < 0.01$). Based on these results, AHBSS was selected as the stimulation pretreatment. Levels of oocyst excystation (which is undesirable) during the 1-h stimulation treatment were determined and were found to be only 1.3% for AHBSS containing 1% trypsin and less than 1.3% for AHBSS alone.

Addition of bile to stimulation and infection media. Figure 3 shows the impact of adding bile to stimulation and infection media at neutral pH in order to mimic pH conditions found in the duodenum. Bile clearly inhibits focus development when it is added at either step.

Optimization of infection media. Figure 4 shows that addition of glucose to the infection media at concentrations greater than the reference concentration (10 mM) influenced the number of infectious foci. At a concentration of 50 mM, a significant increase (for AHBSS pretreatment with 10 mM glucose compared with AHBSS pretreatment with 50 mM glucose in infection media, $P < 0.1$) in the number of foci was noted, but at a higher concentration (300 mM), glucose inhibited infectivity. Other concentrations did not result in significant changes.

Dual detection: sources of oocyst loss during sample processing. To estimate oocyst losses at various steps of the CC-IFA procedure, spent wash solutions were processed using membrane filtration to recover any oocysts present. Figure 5 shows the levels of oocysts recovered from spent wash solutions at different steps during sample processing. As anticipated, the stimulation step wash solutions accounted for only a small portion (3.7% ± 2.4%) of the total oocyst loss compared to the postinfection steps (28.0% ± 4.7%) and the loss on the cell monolayer (39.6% ± 7.0%); 32.5% ± 10% of the oocysts

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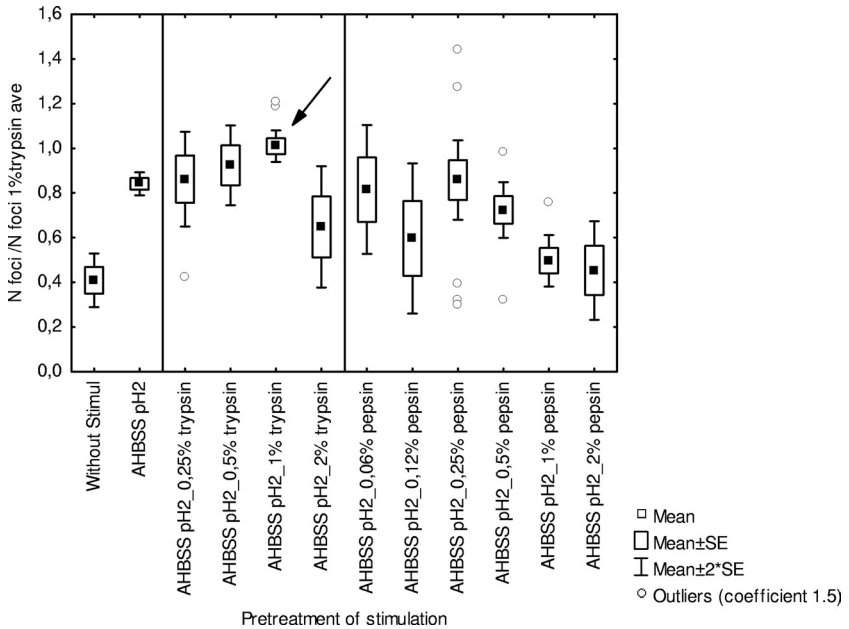

FIG. 2. Impact of addition of trypsin and pepsin in the stimulation pretreatment (AHBSS for 1 h at 37°C) on the number of *C. parvum* cell culture infection foci (combined data from one to five experiments; $n = 3$ to 15). N, number.

were not accounted for. The sources of the losses are not clear, but the losses may well have been the result of the manipulations. Importantly, the variations in these results is low, and therefore the results can be reproduced.

Dual detection: what could influence oocyst adhesion to the cell monolayer? To assess the impact of viability on adherence, CC-IFA was performed with mixtures containing increasing proportions of UV-inactivated oocysts. Figure 6 shows that inactivated oocysts were retained on the cell monolayer to the same extent as untreated oocysts. The decrease in the percentage of infectious foci ranged from approximately 0 to 100% and was proportional to the increasing fraction of UV-inactivated oocysts.

The impact of using low-volume inocula to promote contact of oocysts with cell monolayers was assessed. The use of up to 7 h of incubation with a low inoculum volume (100 μl) before the remaining infection medium (total volume, 500 μl) was

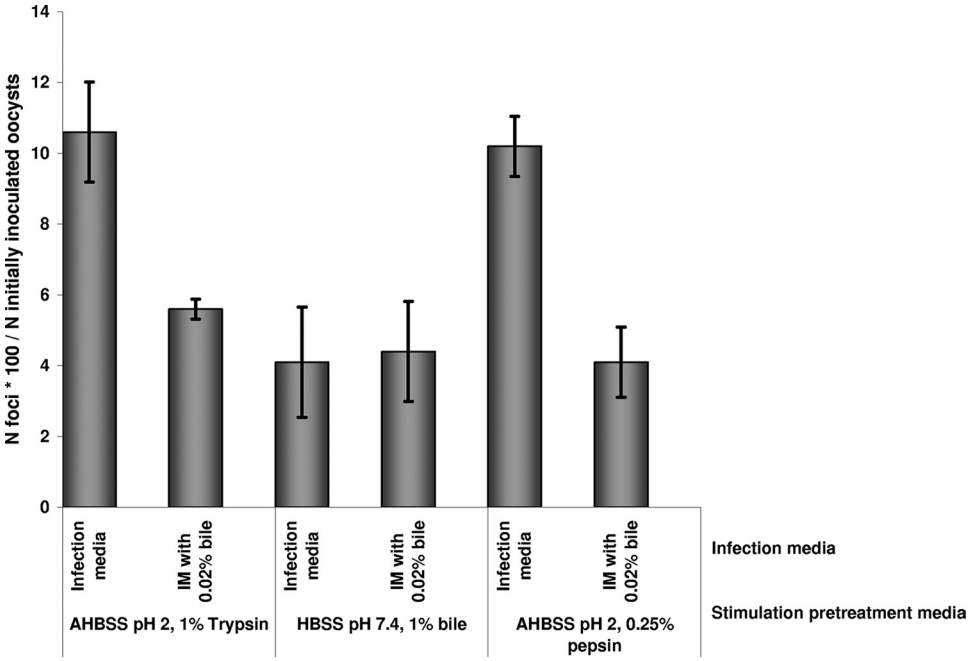

FIG. 3. Impact of addition of bile to the stimulation medium (pH 7.4) and infection medium (IM) on the number of *C. parvum* cell culture infection foci and calculation of the percentages of infectious oocysts in the inocula ($n = 2$). The bars indicate the ranges. N, number.

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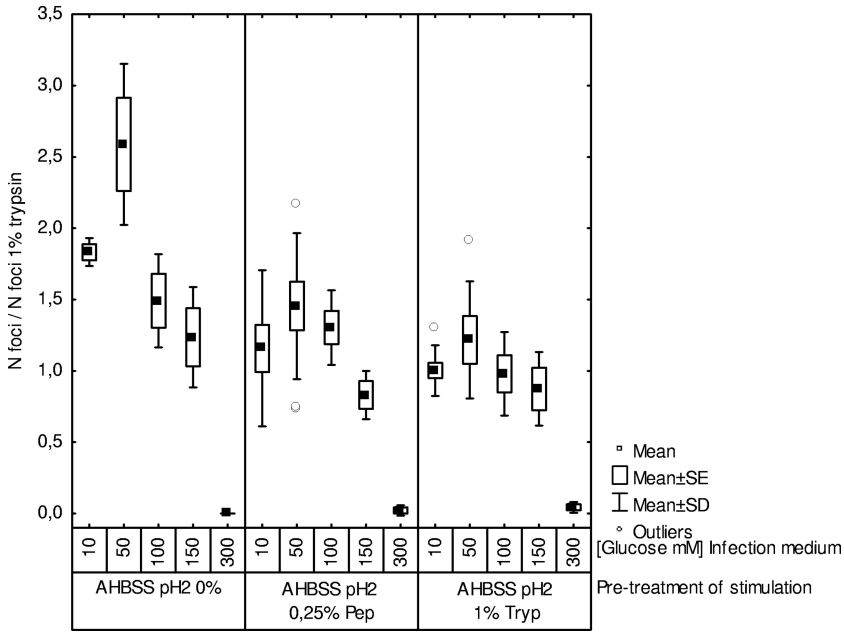

FIG. 4. Effect of glucose concentration on the development of infection foci (combined data from one to five experiments; $n = 3$ to 11). Pep, pepsin; Tryp, trypsin; N, number.

added did not significantly influence ($P \gg 0.1$) oocyst retention (data not shown) or the development of foci. The impact of the incubation time (i.e., 72, 96, and 120 h) on the retention of oocysts on cell monolayers was also investigated, and incubation time was found to not affect oocyst retention on monolayers greatly (data not shown). On the basis of these results, we elected to incorporate estimation of the number of oocysts in postinfection waste streams in the method instead of continuing to attempt to optimize oocyst retention. General microscopic observations of the infected cell monolayers and waste solutions did not reveal any trends in the preferential retention of full or open oocysts or in the simultaneous presence of foci and open or intact oocysts.

Selection of antibodies. For the filtration counting, Crypt-a-Glo (Waterborne Inc.) and GC Combo (BTF) were compared using a reference oocyst suspension that was counted in triplicate. The results showed that there was no significant difference between Crypt-a-Glo (Waterborne Inc.) (437 ± 68 oo-

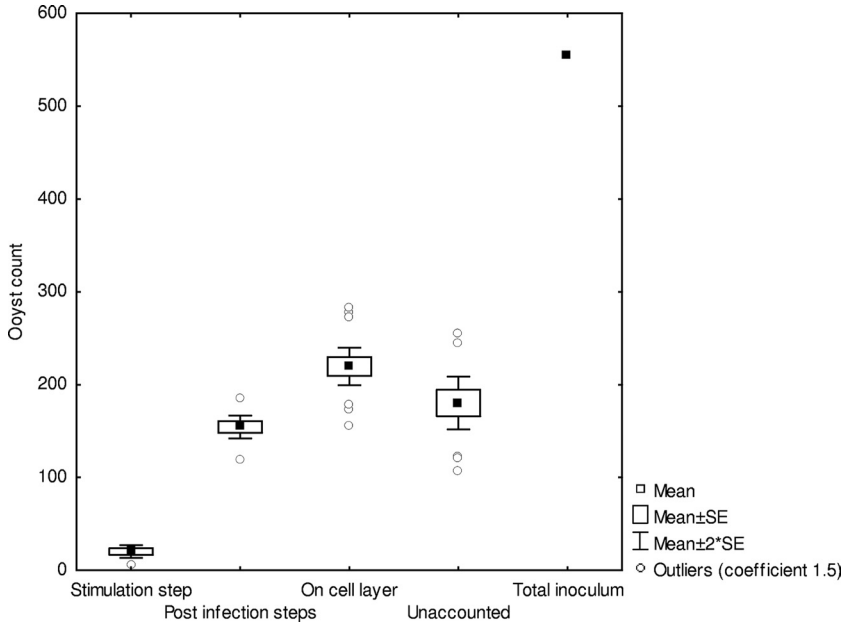

FIG. 5. Recovery of oocysts during processing steps of the CC-IFA method ($n = 15$). For the stimulation and postinfection processing steps oocysts were recovered from spent sample wash solutions by filtration.

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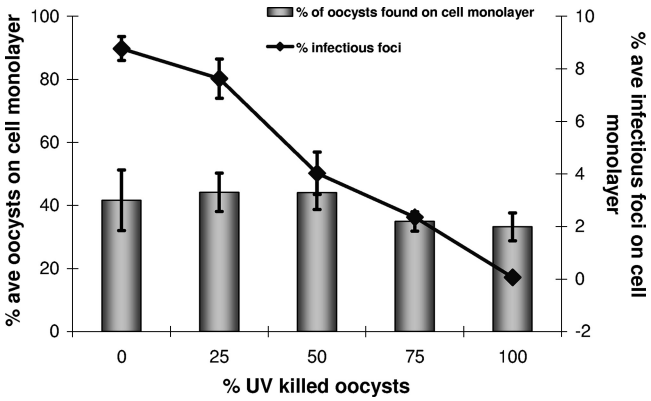

FIG. 6. Adherence of UV-inactivated oocysts (100 mJ/cm^2 with collimated beam) to cell monolayers (n = 3). The errors bars indicate standard deviations.

cysts) and GC Combo (BTF) diluted 1/100 (458 ± 35 oocysts) ($P \gg 0.1$). For detection of oocysts and infection foci on cell monolayers, various antibodies were investigated either alone or in combination. The results demonstrated that Crypt-a-Glo detected both foci and oocysts, while Sporo-Glo and GC Combo detected foci and oocysts, respectively. The lowest level of background staining was observed with the following conditions: 1/10 dilution of Crypt-a-Glo in PBS for 1 h at room temperature; 1/10 dilution of Sporo-Glo in PBS for 1 h at room temperature; and 1/10 dilution of GC Combo in BTF fixing buffer for 1 h at room temperature. Although there was no significant difference in the counts (data not shown), combining Cy3-labeled Sporo-Glo with FITC-labeled GC Combo in BTF fixing buffer facilitated and clarified observation of both oocysts and infection foci (Fig. 7).

3D-CC-IFA recovery studies. Using flow cytometry-sorted oocyst samples, the levels of recovery for 3D-CC-IFA alone were 68% ± 11% (n = 10). The calculated percentage of infectious oocysts in the inocula varied depending on the reference count used, as follows: 12% ± 1% using indirect counts (number of foci/number of inoculated oocysts) and 19% ± 4% based on direct counts of recovered oocysts (number of foci/number of 3D-CC-IFA-counted oocysts) (Fig. 8 and Table 2). For recovery by the 3D-CC-IFA method including upstream IMS, flow-sorted oocysts were spiked into MilliQ water at the IMS step, resulting in levels of recovery of 64% ± 9% (n = 3). For these samples, the percentages of infectivity for indirect calculation were 9% ± 0%, and for direct calculation the percentages of infectivity were 14% ± 2%. For Yamaska River water, levels of IMS and 3D-CC-IFA recovery of 25% ± 19% (n = 3) were observed, and the levels of infectivity varied from 4% ± 3% for the indirect calculation to 19% ± 15% for the direct calculation. For Mille Iles River water, levels of IMS and 3D-CC-IFA recovery of 41% ± 14% (n = 2) were obtained, and the levels of infectivity varied from 5% ± 1% for the indirect calculation to 13% ± 3% for the direct calculation (Fig. 8 and Table 2).

DISCUSSION

Impact of the medium selected on pretreatment. Most workers have obtained stimulation using an acidified buffer amended with 1% trypsin and have indicated that trypsin acts

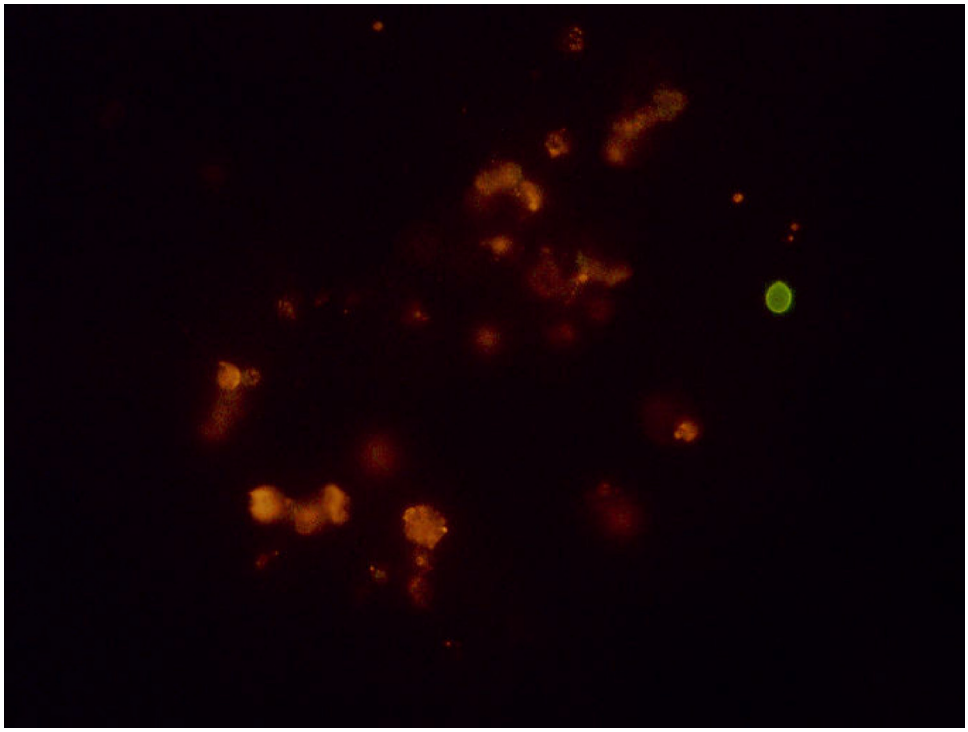

FIG. 7. Superimposed 3D-CC-IFA images (magnification, ×1,000) of a *Cryptosporidium* cell culture infection focus stained with Cy3-labeled Sporo-Glo antibody (red) and an oocyst stained with FITC-labeled GC Combo antibody (green).

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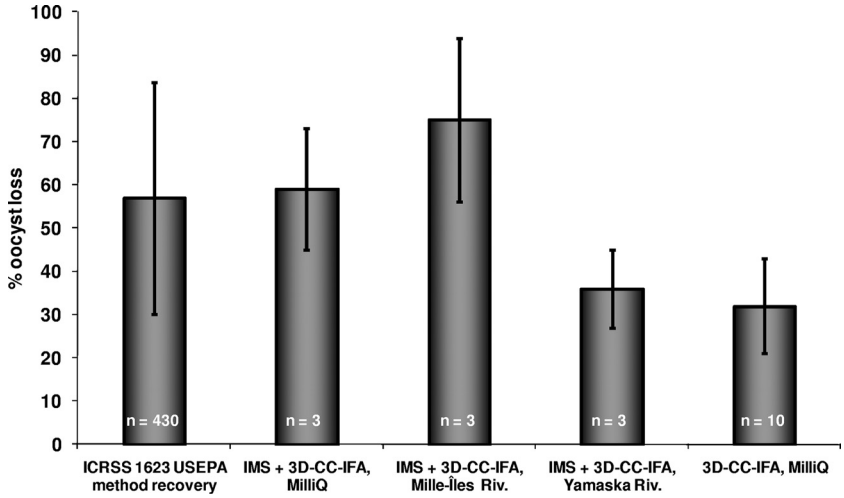

FIG. 8. Comparison of data reported by the USEPA and measured (this study) oocyst loss for steps of USEPA method 1622/3 and of the 3D-CC-IFA method for two different natural waters and MilliQ water using flow cytometry-sorted *C. parvum* oocysts. ICRSS, Information Collection Rule Supplemental Surveys (7). The errors bars indicate standard deviations.

as a proexcystation treatment (20), but the actual benefits of trypsin addition have not been assessed. Trypsin production in the digestive system takes place in the pancreas, and trypsin is secreted through the pancreatic duct into the duodenum at a pH of about 8. Trypsin enzymatic activity peaks at pH 7 to 8 (37), and the activity at pH 6 is less than 7% of the peak activity. A protease active in the stomach at low pH, such as pepsin (2), appears to be a more feasible physiological enzymatic treatment. However, we found that addition of proteases at low concentrations (≤0.5% pepsin and 2% trypsin) did not increase the number focus counts. In fact, acidity was the main stimulation trigger among all of the conditions tested. This is in agreement with a previous study (24) which identified body temperature and stomach acidity (pH 1.5 to 3.5) as the key stimulating factors. The negative impact of a greater concentration of protease (≥1% pepsin and 2% trypsin) was not the result of a pH change, since increasing the protease concentrations did not change the sample pH (≤0.3 pH). Therefore, our results suggest that protease activity is not a significant stimulation trigger. Excystation of *C. parvum* oocysts during cell culture pretreatment was shown to be marginal (≤1.3%), which is desirable for preventing damage to sporozoites during sample processing and inoculation of monolayers. However, only *C. parvum* was tested, and this is an intestinal species. Gastric species, such as *C. muris* and *C. andersoni*, can excyst in both acidic and neutral conditions. Since *C. parvum* and *C. hominis* are both intestinal species and are the major human-pathogenic *Cryptosporidium* species (49), we believe that the low levels of excystation during cell culture pretreatment have a negligible effect on the assay.

TABLE 2. Impact of the reference total oocyst count on the calculated percentage of infectious oocysts in experiments performed at various steps of the 3D-CC-IFA with spikes of flow cytometry-sorted oocysts

Matrix	Steps (*n*)	% Infectious oocytes [avg (SD)][a]		*P*
		Direct	Indirect	
MilliQ	3D-CC-IFA (10)	19 (4)	12 (1)	≪0.001
MilliQ	IMS + 3D-CC-IFA (3)	14 (2)	9 (0)	<0.01
Mille-Iles River	IMS + 3D-CC-IFA (2)	13 (3)	5 (1)	0.062
Yamaska River	IMS + 3D-CC-IFA (3)	19 (15)	4 (3)	0.17

[a] The direct percentages were determined as follows: (number of foci × 100)/(total number of oocyst postinfection steps). And the indirect percentages were determined as follows: (number of foci × 100)/(initial number of oocysts injected).

Addition of bile to the stimulation and infectious media. Bile had little or no effect when it was added to the stimulation or infection media. Stimulation media at pH 7.4 mimicked the physiological conditions of the small intestine, where bile enters the digestive tract. Kato et al. suggested that bile salts could promote excystation (24). It was hypothesized that addition of bile to infection media would enhance oocyst excystation, especially after stimulation by acid. To our surprise, our results showed a clear decrease in the focus counts when bile was added to the infection media. According to another study, sodium taurocholate enhanced the invasiveness of freshly excysted sporozoites by boosting their proteinic secretions and their gliding motility (14).

Impact of medium selection on infection. The impact of addition of glucose to the infection media was investigated by varying the glucose concentration. When 50 mM was added, the infectivity rate increased. This finding is in agreement with the findings of Upton et al., who reported that addition of 50 mM glucose increased the amount of parasite (all life stages) compared to addition of 20 mM (47). However, the counting method and infection criteria of these workers differed (parasite life stage counts versus focus counts), and it is possible that the impact of glucose varies depending on the property assessed (invasion intensity versus infection capacity). These authors also established that addition of galactose, maltose, and mannose increased the number of parasites recovered. Addition of 50 mM glucose to the infection media is also part of the method used in two environmental studies, but the effects of

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glucose addition on focus counts were not investigated (11, 27). In this work, elevated concentrations of glucose (300 mM) in the infection media were found to reduce the number of focus counts. This concentration of glucose is greater than that commonly found in canned fruit juices and juice syrups according to Health Canada (http://205.193.93.51/cnfonline/), and the effect may be due to osmotic effects on sporozoites.

Oocyst loss in dual detection procedure. Enumeration of the total oocysts and enumeration of the infectious oocysts were combined in a single assay (3D-CC-IFA) in order to minimize bias from split or paired (but different) samples. Early work suggested that only about 40% of inoculated oocysts are actually retained on cell monolayers when samples are analyzed to determine the number of infectious oocysts using the CC-IFA method. To fully account for oocyst losses and to determine the modifications of the method required to minimize these losses, a mass balance approach was used, which included counting the oocysts in all sample processing waste streams of the CC-IFA method. This mass balance analysis revealed that the 3D-CC-IFA recovered 68% ± 11% of the seeded flow cytometry-sorted oocysts, about 28% of which were found in postinfection waste streams. In order to avoid filtration of oocysts found in waste streams, several experiments were conducted to facilitate adhesion of oocysts on cell monolayers. However, as described below, these attempts were not fruitful. Other studies are required to streamline the filtration steps in the 3D-CC-IFA method.

Factors affecting oocyst adhesion to the cell monolayer. Addition of trypsin can increase the translucency of the oocyst wall and may degrade, modify, or rearrange the oocyst wall molecules and trigger the adhesion of oocysts (live and dead) on a cell layer (42). However, in our study addition of trypsin (1%) did not influence oocyst adhesion (data not shown), which was likely due to the reduced enzymatic activity of trypsin at low pH. The impact of viability on cell adherence was assessed. A controlled assay using UV-exposed oocysts was performed to verify that nonviable oocysts still adhered to monolayers. It was hypothesized that oocyst viability could be a significant factor influencing adhesion, which is a critical issue for application of the 3D-CC-IFA method since the viability of environmental oocysts varies widely (25). The results indicated that UV inactivation did not influence the number of oocysts retained on a cell monolayer. The effect of low-volume inocula was investigated with the objective of enhancing the contact between the oocysts and the cell monolayer. No significant improvements in oocyst adherence were observed. Other attempts to maximize oocyst adherence, such as addition of trypsin and longer infection times (72, 96, and 120 h), did not improve oocyst adherence to cell monolayers or the numbers of infection foci. Incorporation of the filtration count technique into the 3D-CC-IFA protocol appeared to be the best option for obtaining total oocyst counts and attempting to obtain an oocyst mass balance. The protocol allows recovery of about 70% of seeded oocysts based on trials with flow cytometry-sorted oocysts.

Selection of antibodies for immunodetection by dual detection. Although no statistical difference was observed between the counts obtained with FITC-labeled Crypt-a-Glo alone and the counts obtained with Cy3-labeled Sporo-Glo and FITC-labeled GC Combo together, the latter compounds facilitated and clarified the counts. This combination of compounds is useful for analysts processing environmental samples, especially for differentiation of life stages in samples with some interfering background staining.

Benefits of the 3D-CC-IFA direct count method. The 3D-CC-IFA method with direct counting allows enumeration of total and infectious oocysts for the same sample. For an indirect counting method, the numbers of total and infectious oocysts would have to be estimated by using different samples (or by splitting a sample), resulting in counts which are subject to different sources of losses and compounded errors. Method 1623 sample processing probably causes fewer oocyst losses and errors than the additional sample handling required for the infectivity assay. Therefore, the indirect counting method for estimating infectious oocyst fractions is probably not as accurate as a direct counting method, such as that used for the 3D-CC-IFA method. Estimates of direct and indirect counts for 3D-CC-IFA and for IMS with 3D-CC-IFA were significantly different and highly reproducible when MilliQ water was used ($P < 0.01$), and the differences were marginally significant when the Mille-Iles River matrix was used ($P < 0.07$). In contrast, the high standard deviation for the Yamaska River data prevented detection of any significant difference, but the average estimates were always considerably lower. Moreover, due to the low levels of oocysts in environmental waters, the use of paired samples introduces additional error into the estimates, as previously reported for the detection of infectious and total oocysts. LeChevallier et al. (27) observed that of 22 samples (from a total of 560 paired samples) that tested positive for infectious oocysts using a cell culture PCR infectivity assay (CC-PCR), 21 tested negative for oocysts by method 1623. This type of discrepancy can be expected due to the low levels and distribution of naturally occurring oocysts, as demonstrated by these authors using statistical modeling. Recent comparative trials of infectivity methods (22) have revealed that false-positive results are not common when the CC-IFA method is used. Since the 3D-CC-IFA method uses a direct enumeration approach to obtain total and infectious oocyst counts, it has a significant advantage over indirect methods for analysis of environmental samples with low levels of naturally occurring oocysts.

Levels of recovery for the 3D-CC-IFA with natural and MilliQ waters. The level of recovery is a critical factor to take into account, as it may vary considerably from one water matrix to another. Significant matrix effects have been reported for the cartridge concentration step and the IMS steps for recovery of *Cryptosporidium* from water (7, 32). The proposed method for environmental waters using Envirochek-HV filter concentration, IMS purification, and 3D-CC-IFA detection shares the first two steps with USEPA method 1623. The levels of recovery using method 1623 for analysis of raw surface water samples vary widely and are often low, ranging from near 0% to almost 90% (16, 26, 29, 36). Envirochek filtration and IMS isolation are major contributors to oocyst losses, and they are both recognized by the USEPA and the United Kingdom's Drinking Water Inspectorate (DWI) (12) as procedural steps that result in oocyst loss. Turbidity is often, but not always, pointed to as a major contributor to these variations (16, 26, 32). In the current study, the average levels of 3D-CC-IFA recovery for seeded river water from two different sources were

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25% and 41%, and the turbidities ranged from 10.3 to 13.5 nephelometric turbidity units. The observed contribution of the IMS isolation step to losses was variable and depended on the water matrix, as suggested previously (9, 15). The 3D-CC-IFA method and USEPA method 1623 share the same concentration and purification steps, and the level of recovery for USEPA method 1623 is known to be highly variable, as shown in Fig. 8. The levels of recovery for water sources are shown as examples of applications and are expected to be highly variable depending on the matrix. Compared to typical combined losses resulting from the concentration and IMS separation steps, the typical oocyst losses of 30% with the 3D-CC-IFA method appear to be acceptable, in light of the need to maintain homogeneous losses for determination of the infectious fraction. We believe that once the oocysts are separated, concentrated, and purified, the matrix effect is not significant at the cell culture step. Results obtained using CC-IFA infectivity assessment methods have been shown to correlate with results of animal assays, providing an interesting alternative to animal trials (33, 39).

3D-CC-IFA for various *Cryptosporidium* species. 3D-CC-IFA results and their applications in risk assessment are clearly *Cryptosporidium* species dependent. Given that environmental samples may contain mixtures of oocysts of various species, the impact of different speciation may be threefold: (i) a wide variety of potential infectivity for humans, (ii) different infectivities in cell culture, and (iii) levels of reactivity to detection antibodies. The extent of this impact cannot be fully evaluated in control experiments at this time since most environmentally relevant *Cryptosporidium* species are not readily available. Various environmental species would have to be isolated, purified, and maintained in both cell culture and human trials in order to fully understand these organisms. *C. parvum* and *C. hominis* are known to develop in cell culture (22) and are certainly the major species pathogenic to humans (49), and thus they are the most relevant species for human risk assessment. In terms of the levels of human infectivity, cell culture methods are known to be excellent surrogates for animal infectivity assays (33) when they are used with *C. parvum.* However, it has to be kept in mind that even for the same species, different isolates have significantly different 50% lethal doses in cell culture assays (33), as well as in human trials (6, 13). The potential differences in the levels of reactivity to antibodies can be considered to be low. GC Combo antibody detected many *Cryptosporidium* species in a previous study, including *C. andersoni*, *C. parvum*, genotype W12, *Cryptosporidium* muskrat genotypes I and II, *C. baileyi*, *Cryptosporidium* cervine genotype, and *Cryptosporidium* fox genotype (34). An overriding issue is that the Sporo-Glo antibody, which was developed using *C. parvum* sporozoite antigen, detects *C. parvum*, *C. hominis*, and *C. meleagridis* cell culture clusters of infection but not infection caused by *C. andersoni* or *C. muris* (21; G. D. Di Giovanni, unpublished data). Therefore, the total oocyst count obtained by 3D-CC-IFA includes a broad range of oocysts from both human and animal-associated *Cryptosporidium* spp. and genotypes, but the count of infectious oocysts likely only includes the species with significance for human health.

Conclusion. The combination of total and infectious oocyst counts for a single sample determined using the 3D-CC-IFA method provides an improved estimate of the infectious fraction of oocysts. This method can be used for evaluation of environmental samples, reducing the cost and labor associated with a paired sample approach, and it should provide better data for microbial risk analysis. If combined with the speciation of infection foci (i.e., infectious oocysts) and noninfectious oocysts present in samples, it would provide key information for risk assessment. Currently, the *Cryptosporidium* species shown to infect HCT-8 cells *in vitro* include *C. parvum*, *C. hominis* (22), and *C. muris* (35). Further investigation of other *Cryptosporidium* species using the CC-IFA method could reveal whether this assay is limited in terms of detection of infectious oocysts of other species. Enumeration of total oocysts combined with measurement of the infectious fraction and, in the future, identification of species should provide a sounder basis on which to assess risk and determine treatment requirements. This combination of information should enable the industry to formulate better water treatment strategies and, ultimately, lead to improved protection of public health.

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